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14. ABSTRACT

Using a mouse model of TBI that was established in our lab, we demonstrated that extreme n-3 fatty acid depletion in the mouse brain exacerbates TBI outcome for which a scientific publication in PLoS One was generated. Moderate depletion of DHA (by 30%) in the brain also showed the same trend; TBI-inflicted DHA-deficient mice exhibited slower functional recovery and their brains showed more degradation of alpha spectrin. Both male and female mice were similarly affected by the DHA-depletion. An *in vitro* model to evaluate axon regeneration after injury has been established using cortical neuron cultures. Using this model we demonstrated the induction of axonal regrowth by synaptamide, a bioactive metabolite of DHA. Using quantitative mass spectrometry, the time course of bioactive metabolite production in TBI-inflicted brain was determined. We identified three distinctive groups of metabolites according to the peak time of production after injury. The synaptamide level was steadily increased after TBI. DHA administration protocol is being optimized for the formation of active metabolites such as synaptamide as well as functional recovery outcome after TBI.

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Introduction

Military personnel in combat deployments are afflicted with high rates of traumatic brain injury (TBI) causing lifelong neurological and cognitive impairments, especially in learning and memory. Numerous studies have shown that docosahexaenoic acid (DHA) is essential for proper brain development and function [1,2], although the underlying mechanisms are still unfolding. Under normal conditions, DHA is present in esterified form in membrane phospholipids, especially the aminophospholipids, phosphatidylethanolamine (PE), and phosphatidylserine (PS). Despite tight regulation to maintain membrane phospholipid homeostasis, DHA enrichment can expand the PS pool in neuronal membranes [3], as DHA-containing phospholipids serve as the most favored substrate for PS biosynthesis in mammalian tissues [4]. On the contrary, depletion of DHA has been shown to decrease PS levels significantly in brain tissues [3, 5-7]. Since PS is known to participate in key signaling events supporting cell survival and differentiation, DHA-dependent PS modulation is an important aspect of neuroprotection [8]. Following brain injury, polyunsaturated fatty acids including DHA and arachidonic acid (AA, 20:4n-6) are released from neural membranes and metabolized to many bioactive derivatives. Some of the AA-derived eicosanoids are known to be pro-inflammatory, exacerbating the initial injury [9,10]. In contrast, some DHA-derived docosanoids has been shown to ameliorate or resolve inflammatory processes [11]. Furthermore, N-docosahexaenoylethanolamide (synaptamide), a DHA metabolite of a separate class, has been recently identified as a potent neuritogenic and synaptogenic agent [12]. In this regard, the DHA content of the brain may be an important variable to consider in devising a strategy to improve neuroprotection and recovery outcome after brain injuries.

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Objective

- The major goals of this project are to develop strategies to improve neural resilience to traumatic brain injury and facilitate recovery through mechanism-based optimization of the nutritional DHA or metabolite status in neuronal tissues.

- Aim 1. To determine if diets rich in DHA afford protection to the nervous system against traumatic brain injury in animal models
- Aim 2. To identify bioactive DHA metabolites formed in the brain that are involved in neuronal survival, neurite development, learning and memory
- Aim 3. To determine if DHA-derived mediators improve recovery after traumatic brain injury in animal models
- Aim 4. To devise therapeutic approaches for improving DHA status and/or administering specific bioactive metabolites that facilitate recovery from traumatic brain injury.

Statement of Work

Year 3

Task1: Testing therapeutic potential of DHA and/or DHA metabolites administration on recovery after TBI (months 25-30)
Task 2: Analyzing active metabolites in the control and posttraumatic brains during the course of recovery after dietary manipulation of DHA status or DHA administration (months 18-36)
Task 3: Optimizing the DHA administration protocol (months 31-36)

- Milestone 1: DHA's therapeutic potential tested.
- Milestone 2: *In vivo* formation of active DHA metabolites identified.
- Milestone 3: relationship between the local DHA (or DHA metabolite) status and injury outcome established
- Milestone 4: Publication 2 on effects of DHA status on injury outcome with possible publications on therapeutic effects of DHA and DHA metabolites on traumatic brain injury.

- Milestone 4: Publication 1 on identification of brain DHA-metabolites by isotope-assisted metabolomics approach (Year2)

Report

Task 1: Testing therapeutic potential of DHA and/or DHA metabolites administration on recovery after TBI (months 25-30)

During this report period, we evaluated the time course profile of DHA in blood in preparation of testing the therapeutic potential of DHA and to optimize the DHA administration protocol. Prior to that, we established an adverse impact of moderate DHA-depletion on the functional recovery since moderate depletion is rather commonly observed with the western dietary practice. In addition, we demonstrated deleterious effects of DHA-depletion in both genders.

During the last report period, we successfully generated mice with varying degrees of DHA depletion in the brain by feeding an omega-3 deficient special diet for one to three consecutive generations. We established that DHA-adequate mice recover significantly better from TBI compared to the DHA-deficient mice in the extreme case of DHA-depletion (over 70%). Histological and biochemical measures such as NeuN positive cells and spectrin alpha cleavage also consistently indicated that DHA-adequate mice recover better from TBI. Our findings from mice with extreme DHA-deficiency have been published in PLoS One during this period. To address the situation rather commonly observed with Western dietary practice, we extended our investigation to moderate DHA-depletion (by 30%). Although the extent was not as severe as the extreme case of DHA-depletion, both vestibulomotor functions assessed by rotarod and beam walk tests as well as memory evaluated by the novel object recognition test indicated significant differences between adequate and deficient groups with the latter showing slower recovery. In addition, cognitive function and anxious behavior were also adversely affected by the moderate omega-3 fatty acid deficiency in TBI-inflicted mice. Modest increases in the level of cleaved alpha spectrin II in the cortex of DHA-deficient injured mice as compared to the DHA-adequate controls also supported the adverse impact of moderate DHA-depletion.

Above observations were made in male mice. Even though females constitute of about half the total population, females are seldom used as experimental models because of the possibility that hormonal fluctuations may confound the experimental results. We moderately depleted the brain DHA content in female mice by diet and assessed the recovery after TBI. We found that there is a similar adverse impact of DHA depletion on recovery in terms of motor deficits assessed by beam walk test (Fig. 1) and memory assessed by fear conditioning. These experiments imply that DHA depletion may have deleterious effects irrespective of gender.

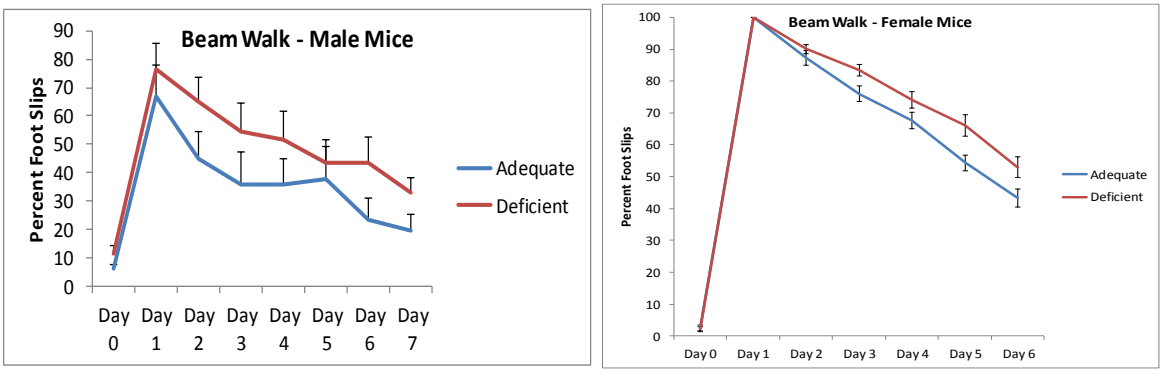

Fig. 1. Adverse impact of brain DHA depletion on the functional recovery after TBI irrespective of gender.

During this period, we started testing therapeutic potential of DHA and the DHA metabolite synaptamide. To ascertain the peak blood levels of DHA after oral or intraperitoneal (i.p.) administration, DHA was dissolved in 5% solutol plus 5% dimethylacetamide in saline or water (for i.p. or oral gavage at 25 mg/kg i.p. or 500 mg/kg, respectively). Blood was collected at 0, 1, 2 and 4 hours after DHA administration and lipids were extracted and analyzed. Both i.p. and oral administration produced similar serum DHA concentration profiles with peak at 1 h

after administration (Fig. 2). Using this protocol, we are in the process of testing the effects of DHA administration on TBI outcome.

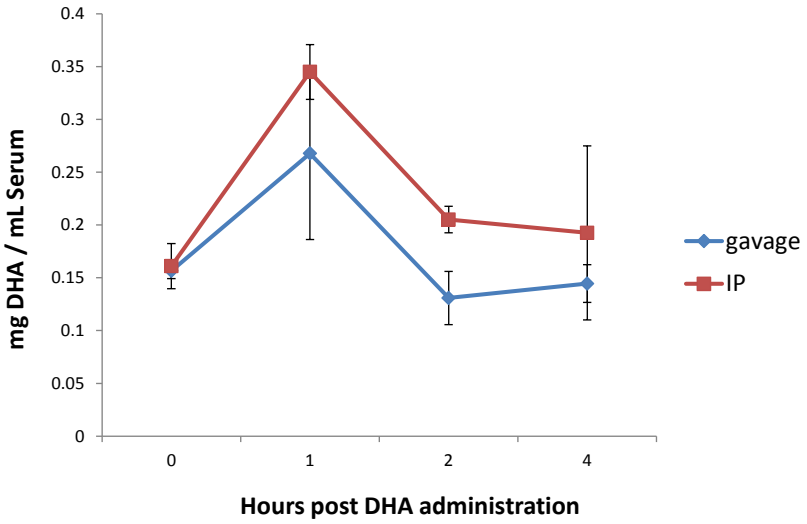

Fig. 2. Time course of the serum DHA level for gavage or i.p. administration of DHA

We have previously identified *N*-docosahexaenoylethanolamine (synaptamide) as a potent neuritogenic, synaptogenic and neuritogenic metabolite of DHA formed in hippocampal and cortical neuronal cultures. To test the effects of synaptamide on axon repair, we have also examined the regrowth of axons after injury during this review period. We first established an *in vitro* axon injury model using a microfluidic culture platform. In this model, cortical neurons were seeded in one side of a culture chamber and axons were allowed to grow through multiple grooves to reach the other side of the chamber. At the end of the grooves (dotted line), axons were severed by rapid aspiration of the media from the axonal compartment, and regrowth of axon was monitored using the axon specific marker SMI-312. This model allowed us to find that synaptamide at a concentration as low as 10 nM stimulated axon regrowth in axotomized cortical neurons (Fig. 3), indicating the potential of synaptamide for axonal repair after injury.

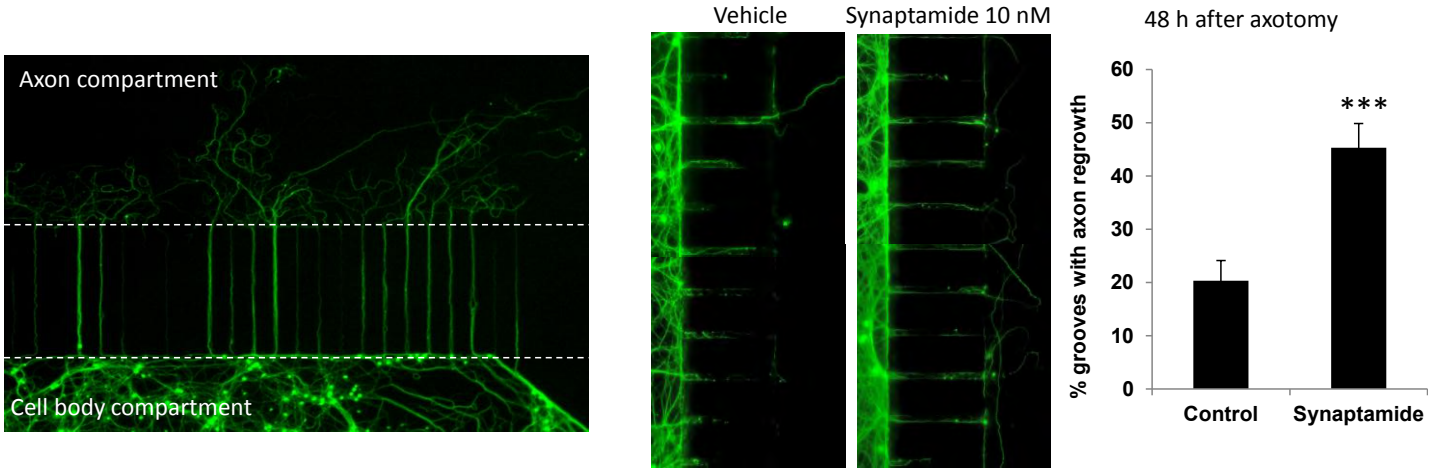

Fig. 3. Regrowth of axon (SMI-312 positive, green) evaluated in an axon device after 14 days *in vitro* culture of cortical neurons followed by axotomy and synaptamide treatment.

Task 2: Analyzing active metabolites in the control and posttraumatic brains during the course of recovery after dietary manipulation of DHA status or DHA administration (months 18-36)

Using the mass spectrometric analysis method that we established, we measured the TBI-induced metabolite formation time course during this period. We found that there are three distinctive classes of metabolites in

terms of the peak production time. This information will enable us to optimize the DHA or metabolite injection time to improve TBI outcome.

The C57BL/6N male mice were injured by CCI delivered after craniotomy to the left hemisphere of the brain. The mice were euthanized at specific time intervals after TBI by cervical decapitation. The brain was rapidly removed and the cortex around the injury site and hippocampus from the injured hemisphere and the corresponding parts from the uninjured hemisphere were dissected, immediately frozen in a dry ice/isopropanol slurry and stored at -80° C until analysis. Tissues were homogenized and lipids extracted via reverse phase solid phase extraction in the presence of a mixture of deuterated internal standards. The extract was analyzed via HPLC-MS and HPLC-MS/MS in the negative ion mode. Metabolites were identified by MS/MS and with the help of corresponding internal standards when they are available. Levels of metabolites as a function of time after TBI were evaluated by comparison of peak areas with internal standards using either the [M-H]- or a unique fragment ion. Quantitation of the metabolites for which the internal standard of identical structure was not available was based on a relative term.

We found three distinctive patterns in the time course of the TBI-induced metabolite formation. Prior to injury, synaptamide and anandamide levels in the cortex were about 0.01 fmol/µg protein. At 1 h after TBI, synaptamide levels in the injured cortex increased to 0.3 fmol/µg protein. Anandamide also increased to about 0.3 fmol/µg protein in the same period. Both synaptamide and anandamide levels increased through 48 h post-injury to 8.9 and 3.0 fmol/µg protein, respectively (Fig. 4a). It has not been tested whether their levels would continue to rise past 48 hours. Although the total DHA and AA content was found to be about 15 and 10 mole %, respectively, the level of free AA is significantly greater than that of DHA at time points examined (Fig. 4b). For example, the AA and DHA levels increased from about 2 and 0.2 pmol/µg protein at the basal condition to 3.4 and 2.6 pmol/µg protein at 48 h after TBI, respectively. Nevertheless, the synaptamide level after TBI is significantly higher than anandamide, suggesting preferential synthesis of synaptamide after TBI.

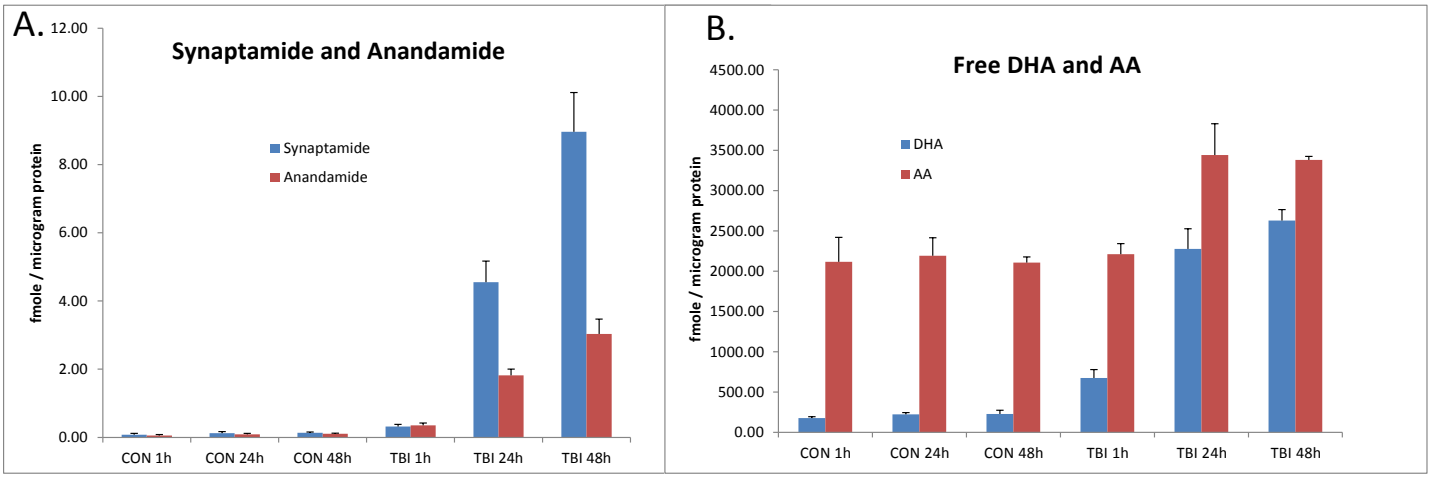

Fig. 4. Time course of TBI-induced formation of fatty acid ethanolamides (A) and free fatty acids (B).

Cyclooxygenase metabolites of AA including thromboxane and prostaglandins showed a more acute time course. Thromboxane B2 (TXB2), PGF2-alpha and PGD2/E2 peaked at 1 h, followed by a downward trend at 3 and 6 h after TBI (data not shown) to about baseline by 24 h after injury (Fig. 5). Most monohydroxy derivatives of both DHA and AA (HDoHE and HETE) except 12- and 15-lipoxygenase products increased within 1 h after TBI, reached close to peak values by 24 h and remained highly elevated through 48 h (Fig. 6). The level of 12- and 15-lipoxygenase products 14- and 17-HDoHE as well as 12-HETE peaked at 24 h and decreased significantly by 48 h (Fig. 7). The indicated time course of specific metabolites will be the basis for selecting optimum DHA administration time points for testing therapeutic potential in the extended budget period. In addition, we are in the process of measuring the active metabolite status in DHA-adequate and deficient animals where significant differences in functional recovery outcome have been demonstrated.

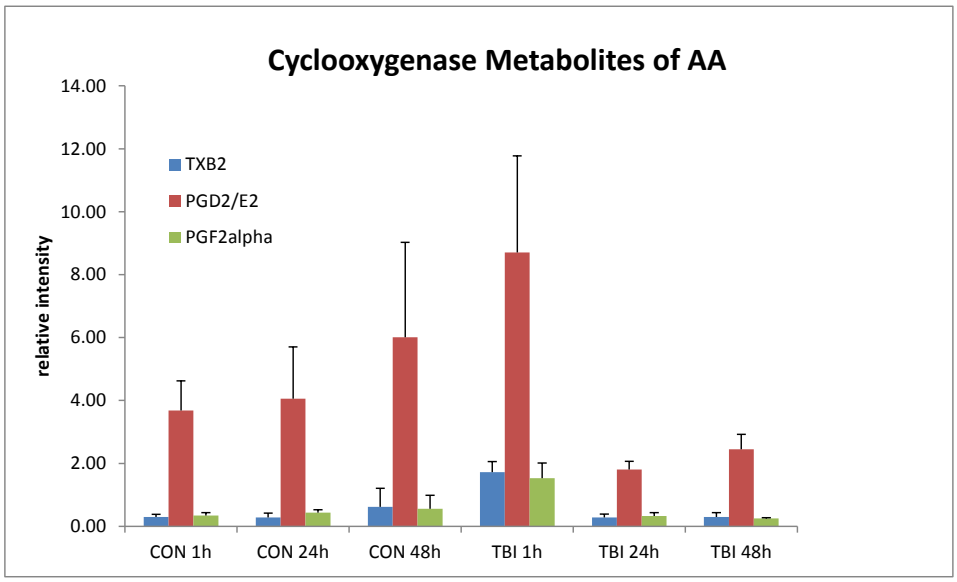

Fig. 5. Time course of TBI-induced formation of cyclooxygenase products of AA.

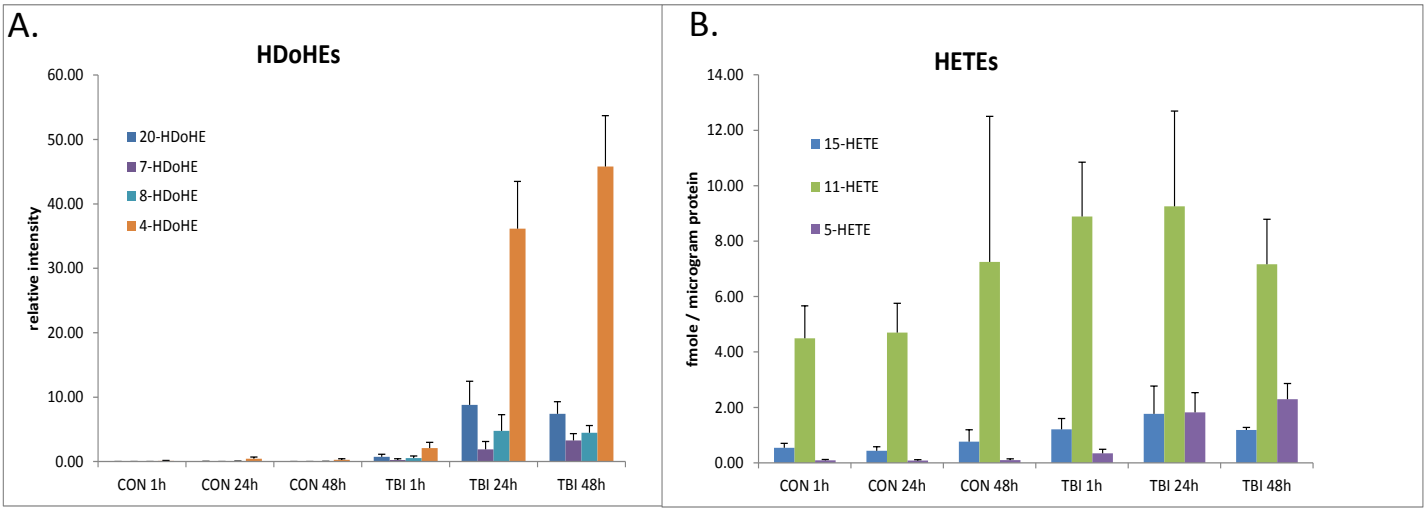

Fig. 6. Time course of TBI-induced formation of DHA- (A) and AA-derived (B) monohydroxy products.

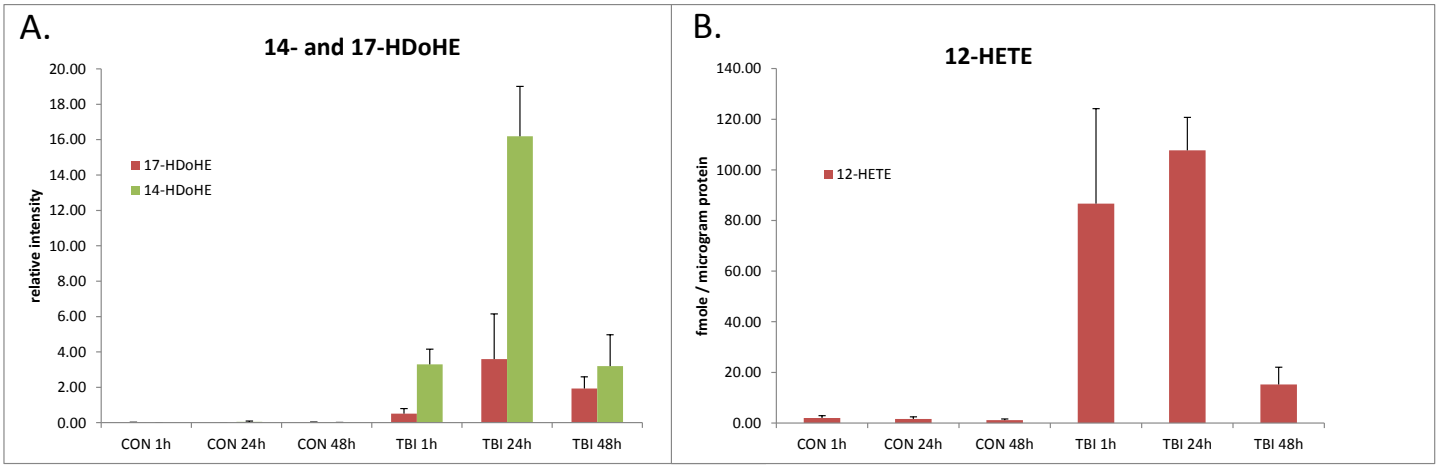

Fig. 7. Time course of TBI-induced formation of 12- and 15-lipoxygenase products of DHA (A) and AA (B).

Task 3: Optimizing the DHA administration protocol (months 31-36)

We are in the process of testing the effects of DHA administration on functional recovery. We are still optimizing the administration protocol in terms of administration rout, time and dose as well as diet selection after injury. We will accomplish this during the no cost extension period.

Key Research Accomplishments

1. Using a mouse model of TBI, we established the adverse impact of moderate DHA-depletion on spontaneous recovery from injury.
2. We demonstrated the adverse effects of DHA-depletion in both genders.
3. Using an axon growth model using a microfluidic culture platform, an *in vitro* injury model was established. Preliminary results indicate-stimulated axon regrowth with synaptamide treatment
4. The time course of TBI-induced metabolite formation from DHA and AA was determined.

Reportable Outcomes

A paper entitled "Depletion of Brain Docosahexaenoic Acid Impairs Recovery from Traumatic Brain Injury" has been published in PLoS One.

Conclusion

- Milestone 1: DHA's therapeutic potential is being tested.
- Milestone 2: *In vivo* formation of active DHA metabolites have been identified.
- Milestone 3: Relationship between the local DHA (or DHA metabolite) status and injury outcome is being tested.
- Milestone 4: A paper entitled "Depletion of Brain Docosahexaenoic Acid Impairs Recovery from Traumatic Brain Injury" has been published in PLoS One.
- Milestone 4: Manuscript on identification of brain DHA-metabolites by isotope-assisted metabolomics approach (Year2) is now in preparation

During the third year, we have met the above specific milestones approved by the CDMRP. We published a paper on the effect of the DHA status on TBI outcome in PLoS One. Due to delayed installation of the instrument, it was not possible to make the publication 1 on "identification of brain DHA-metabolites by isotope-assisted metabolomics approach" in the second year. However, we were able to establish a method and now a manuscript is being prepared for communication. Using quantitative mass spectrometric approaches we identified increased formation of bioactive DHA and AA metabolites after TBI. We established an axon injury model by which bioactivity of synaptamide was further extended to axon regeneration. We have demonstrated that moderate DHA-depletion has an adverse impact on functional recovery from TBI, as is the case with extreme depletion regardless of the gender. We will continue to pursue the therapeutic dose and time windows of DHA and synaptamide treatment using FAAH KO and wild type animals.